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X-cells in fish pseudotumors are parasitic protozoans

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ABSTRACT: Bottom-dwelling teleosts, particularly flatfishes or cod living in temperate to cold seawater, sometimes develop tumor-like lesions on the body surface or in the branchial cavity. These lesions usually contain masses of so called 'X-cells' of unknown origin. We amplified a gene for small subunit ribosomal RNA (18S rRNA) from X-cell lesions of the flathead flounder *Hippoglossoides dubius*. Phylogenetic analysis clearly classified the obtained sequence as a protozoan, although the organism had no clear affinity with any known protistan groups. *In situ* hybridization showed that probes specific for the protozoan 18S rRNA hybridized only with X-cells, and not with the host-fish cells, indicating that X-cells harbor the protozoan rRNA. On the other hand, a probe specific for vertebrate 18S rRNA hybridized with the host-fish cells, but not with X-cells. This is conclusive evidence that X-cells are parasitic protozoans.

KEY WORDS: X-cell · 18S rRNA · *In situ* hybridization · Protozoa · *Hippoglossoides dubius* · Fishes · Pseudotumor

INTRODUCTION

X-cells are often large, polygonal cells, each of which has a large nucleus and a prominent nucleolus (Brooks et al. 1969). Numerous X-cells are found in tumor-like lesions of various bottom-dwelling fish species, and the 'X-cell disease' has been reported from all over the world; e.g. dab (McVicar et al. 1987) and cod (Dethlefsen et al. 1996) in the North Atlantic, cod and walleye pollock (McCain et al. 1979) in the North Pacific, flatfishes and a gobiid fish (Katsura et al. 1984) in the North Pacific, a catfish in a lake in the USA (Diamant et al. 1994), and a notothenid fish in the Antarctic (Franklin et al. 1993). The tumor-like lesions of the disease were once thought to be related to water pollution (Stich et al. 1976, Wellings et al. 1976). However, no data that link X-cell disease conclusively with pollution have been reported. Viral etiology of X-cells has also been suggested (Peters et al. 1981), based on the facts that virus-like particles have been observed in X-cells (Wellings & Chuinard 1964), and/or that 'nuclear inclusions' in X-cells are morphologically similar to those in cells infected with lymphocystis virus (Peters et al. 1983). However, attempts to transmit the X-cell disease to healthy fishes, or to isolate the possible causative virus by cell culture have been unsuccessful (Wellings et al. 1976, Diamant et al. 1988). On the other hand, Dawe (1981) suggested that X-cells were unicellular parasites, and presented the following data: (1) The amount of DNA of an X-cell was only one-third of that of a cell of the 'host' fish; (2) isozyme experiments showed that some enzyme bands were present only in lesions containing X-cells, not in any other fish tissues; (3) mitotic features of X-cells resembled those of certain protozoan species morphologically. However, despite many reports of this disease over the past 30 yr, the true identity of X-cells is still uncertain (Noga 2000), although recent theories seem to favor protozoan etiology of the disease (Grizzle & Goodwin 1998). Recently, Khattra et al. (2000) detected a ribosomal RNA gene belonging to a hitherto unknown microsporidian in X-cell lesions of English sole, but could not determine whether the X-cells were the microsporidian or not.

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In 2001 and 2002, many flathead flounder *Hippoglossoides dubius* caught by bottom-trawling in coastal waters of Niigata and Yamagata prefectures of Japan (eastern part of the Japan Sea), were found to have tumor-like lesions of the skin. The prevalence of the diseased fish ranged from 0 to 40% of the total catch, and histological examination revealed X-cells in the tumorous lesions. X-cell disease in the flathead flounder had already been reported (Ito et al. 1976, Katsura et al. 1984), although the disease had not previously been recorded in this area. In the present study, we examined the X-cell disease in the flathead flounder to clarify the origin of the X-cells.

MATERIALS AND METHODS

Histological examination. The flathead flounder used for general histological observations were caught by bottom-trawling and landed at a nearby port. Tissues of the lesions were taken from 10 affected fish, and fixed in Davidson's solution (330 ml 95% ethanol, 220 ml commercial formaldehyde solution containing 37 to 39% formaldehyde, 115 ml glacial acetic acid, and 335 ml distilled water). The tissues were then embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin (HE).

DNA extraction and *in situ* hybridization. Immediately after capture with a bottom trawl, tissues of tumor-like lesions in flathead flounder were sampled on board the fishing vessel. Tissues were taken from the lesions of 3 affected flounder. For DNA extraction, excised tissues of the lesions were fixed and stored in 100% ethanol. For *in situ* hybridization, tissues of the lesions were fixed in Davidson's solution overnight, and incubated in 300 mM EDTA (pH 7.5) for 1 wk to decalcify bones or scales in the tissues. From 1 affected fish, pieces of liver, spleen, and kidney were also sampled and fixed as described above. Subsequently, the tissues were embedded in paraffin.

PCR and phylogenetic analysis. DNA was extracted from the ethanol-fixed tissues of the lesions using proteinase-K and phenol-chloroform (Sambrook et al. 1989). The gene for small subunit ribosomal RNA (18S rRNA) was amplified by PCR using the universal primers for eukaryotes, 5′-CGACAACCTGGTTGATCCTGCCAGT-3′ and 5′-TTGATCCTTCTGCAGGTTCACCTAC-3′. Cycling conditions for the PCR were 94°C (30 s), 55°C (30 s, and 72°C (1 min) for 30 cycles. The amplified fragment was purified, ligated with the plasmid vector pDrive (QIAGEN) and applied to nucleotide sequencing. The determined sequence and the 18S rRNA sequences of various eukaryotes were aligned manually. Evolutionary distances were calculated by the method of Kimura's 2-parameter model. For the phylogenetic analysis we used the Genetic software package (Version 10.6, GENETYX, Tokyo).

Probes for *in situ* hybridization. We synthesized 3 antisense oligonucleotide probes complementary to a variable region unique to the obtained sequence so that they would hybridize with the 18S rRNA in the cytoplasm. They were designated AXC1 (5′-CAAGTTCTGAGGCAGTGATTAGCTTG-3′), AXC2 (5′-CTGAATCTTGAACTTAGGCTTAGGGA-3′) and AXC3 (5′-TCGCGAAACGTCCCTGGAGAAATTAA-3′). An oligonucleotide designated TL1 (5′-AGAGCATCGAGGGGGCGCCGAGAGGC-3′), which would hybridize with the 18S rRNA of the flathead flounder (and presumably with the 18S rRNA of other vertebrates) was also synthesized. For a negative control, a mixture of 4 oligonucleotides specific to the 16S rRNA of the bacterium *Flavobacterium psychrophilum* were used: 5′-ATAGTGTGTTGATGCCAACTCACTATAC-3′, 5′-GTCAAGCTACCTCACGAGGTAGTGTTTC-3′, 5′-CGATCCTACTTGCGTAG-3′, and 5′-AGTGTGTTGATGCCAAC-3′.

Probes were labelled with digoxigenin using a commercial kit (DIG Oligonucleotide Tailing Kit, Roche) according to the manufacturer's description.

***In situ* hybridization.** Experiments were carried out at room temperature unless otherwise indicated. Paraffin sections were cut at 3 µm, deparaffinized and rehydrated. These sections were treated with 10 µg ml^{-1} proteinase-K in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, at 37°C for 15 min. After rinsing the slides in 10 mM phosphate-buffered saline (pH 7.5), they were incubated for 30 min with prehybridization solution. Subsequently, the sections were incubated with hybridization solution, covered with Hybri-Slips (Sigma-Aldrich, Germany) in a humid chamber in an incubator at 60°C for 1 h. The temperature setting was then changed to 39°C without opening the incubator, thus allowing the incubation temperature to decrease gradually. The slides were continously incubated overnight. The hybridization solution was prepared by adding probes and dextran sulphate to the pre-hybridization solution. The final hybridization solution contained 21.4 mM Tris-HCl pH 7.6, 0.5 mg ml^{-1} sheared salmon sperm DNA (Eppendorf AG, Germany), 80 µg ml^{-1} polyadenylic acid (Roche Diagnostics, Germany), 4 µg ml^{-1} poly-deoxy-adenylic acid (Roche), 1 × Denhardt's solution (Wako Pure Chemical Industries, Japan), 10% dextran sulphate, 1.07 M NaCl, 6.4 mM EDTA, and 10 pmol ml^{-1}-labelled probes. After incubation, the slides were washed in 2 × SSC (standard saline citrate; 1 × SSC contains 150 mM NaCl and 15 mM $Na_3C_6H_5O_7$, pH 7.0) at 39°C for 5 min twice and in 1 × SSC at 39°C for 5 min twice. The slides were then rinsed in 100 mM Tris buffer at pH 7.5 containing 0.88% NaCl, and the signal was detected

immunohistochemically with peroxidase (PO)-labelled anti-digoxigenin sheep antibody (Fab fragments, Roche). Final visualization of the signal was performed with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride, following which the sections were weakly counterstained with hematoxylin. For some sections, signal detection was also carried out with alkaline phosphatase (AP)-labelled anti-digoxigenin sheep antibody (Fab fragments, Roche) and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche), which yielded principally the same results as with the immunohistochemistry test using the PO-labelled antibody. However, the resultant color deposition with the AP reaction was much more diffused, and hence immunohistochemistry with PO-labelled antibody was mainly adopted in the present study.

RESULTS

The affected fish had developed lesions on their ocular (eyed) side or fins (Fig.1a). We observed 2 types of lesions in diseased fish: (1) papilloma-like, soft outgrowths of the skin; polyp-like, the outgrowths were sometimes connected to the skin by thin stalks; some were ulcerated. (2) Fibroma-like, fairly hard, small outgrowths of the skin, filled with homogenous fleshy white tissue. Type 2 lesion was less frequent than Type 1. No particular abnormality was observed in the visceral organs.

Under light microscopy, the papilloma-like lesions appeared very similar to epithelial tumors, with many complex papillae in the epithelial folds (Fig. 1b). Higher magnification revealed numerous X-cells in all lesions examined. In the epidermis, X-cells were located mainly in the basal part of the epithelium. The morphology of the X-cells in the epithelium was almost the same as that described by many researchers for X-cell disease in other fish species (Fig. 1c): they were often large and eosinophilic, and each X-cell had a centrally-located, large nucleus with a prominent nucleolus. The nuclei of X-cells stained only weakly with hematoxylin. In severe cases, granulation tissue had developed between the papilloma-like epithelium and the dermis and contained numerous much smaller X-cells. These small X-cells were stained with eosin, and also had centrally located nuclei with prominent nucleoli. The size of the small X-cells was comparable to that of migrating leucocytes of the flounder. X-cells of intermediate size were observed both in the granulation tissue and in the epidermis. The fleshy tissue of the fibroma-like outgrowths consisted of granulation tissue formed in the swollen dermis (Fig. 1d), and contained numerous small X-cells. The epithelium of these lesions had disappeared. In all lesions examined,

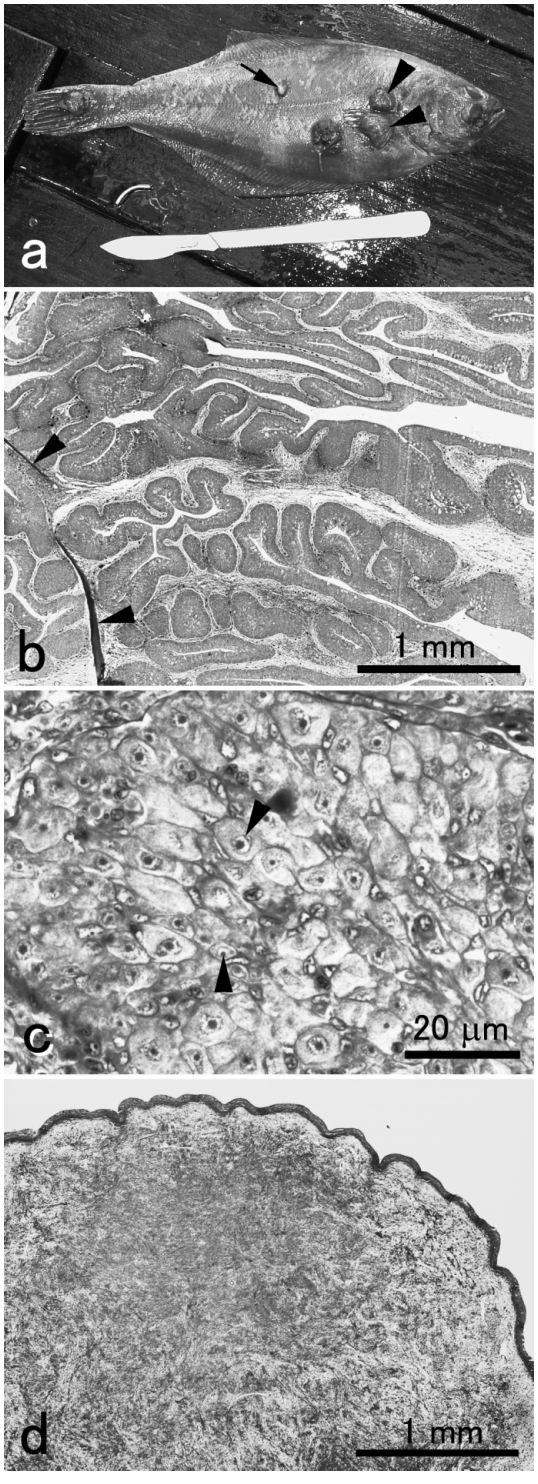

Fig. 1. *Hippoglossoides dubius*. (a) A flathead flounder, affected with X-cell pseudotumors, note papilloma-like lesions (arrowheads) and fibroma-like lesion (arrow) on skin and fins; scalpel length = 15.5 cm. (b) Section of papilloma-like lesion, arrowheads indicate scales (hematoxylin-eosin, HE). (c) Papilloma-like lesion in (b) at higher magnification, showing X-cells of various sizes (arrowheads). (HE). (d) Fibroma-like lesion (HE)

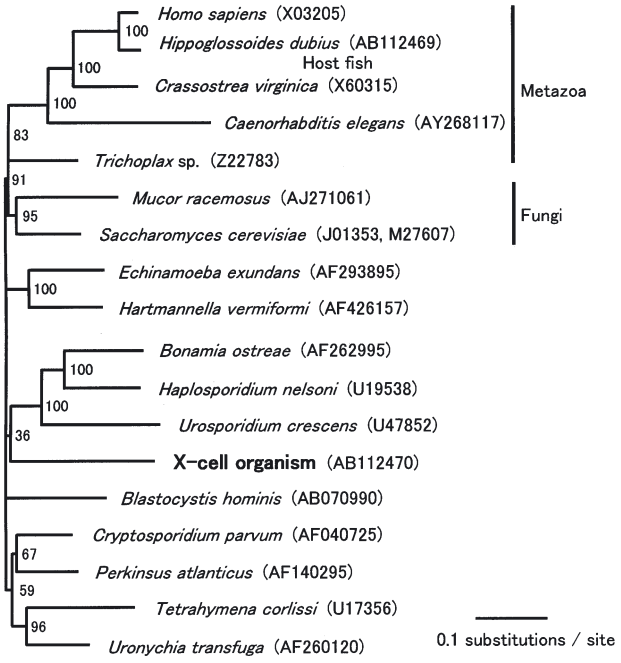

Fig. 2. Phylogenetic relationships of X-cell organism among eukaryotes, inferred from 18S rRNA gene sequences by neighbor-joining method. Bootstrap probabilities are shown on internal branches (%). Database accession numbers indicated in parentheses

X-cells were present only in the skin, including the dermis and epidermis; no X-cells were observed in the underlying muscle tissue.

Using the universal primers for eukaryote 18S rRNA, a gene was amplified by PCR. The phylogenetic analysis clearly classified the determined sequence as a protozoan (DDBJ Accession no. AB112470). However, this organism had no clear affinity with any known protozoan group, forming only a weak cluster with haplosporidians (Fig. 2). The sequence was also distinct from the sequence detected in X-cell disease of English sole by Khattra et al. (2000) (GenBank Accession no. AF201911), and showed no affinity with microsporidians.

In situ hybridization with the probes for the protozoan 18S rRNA gene revealed that only X-cells, and no presumed fish cells, contained the protozoan 18S rRNA (Fig. 3). All types of X-cells visible in the HE-stained sections hybridized positively with these probes. *In situ* hybridization with the probe designed to hybridize with fish or vertebrate 18S rRNA produced a pattern completely complementary to that produced by *in situ* hybridization using the probes for the 18S rRNA of X-cells. Only host-fish cells, and no X-cells, contained vertebrate 18S rRNA (Fig. 3). Numerous X-cells were also detected in fibroma-like lesions (Fig. 4a). In some lesions, X-cells were present only in the basal parts of the hypertrophied epidermis (Fig. 4b). *In situ* hybridization occasionally revealed a few X-cells in visceral organs such as the liver or kidney, but the cells did not seem to proliferate in these organs.

DISCUSSION

The results of the present study provide conclusive evidence that X-cells in fish pseudotumors are parasitic protozoans, at least in the flathead flounder *Hippoglossoides dubius*. Because of the similarity in the morphology of dividing cells, Dawe (1981) suggested that the X-cell was a member of the protozoan family Hartmannellidae. However, the present phylogenetic analysis revealed no particular affinity of the X-cell organism with Hartmannellidae. The sequence of 18S rRNA in the X-cell organism is also clearly different from that of microsporidians. In English sole affected by X-cell disease, Khattra et al. (2000) detected an rRNA gene that belonged definitively to a microsporidian. However, it is likely that these investigators detected the gene of a microsporidian that coexisted with the X-cell organism in the sole since, although X-cells have been found in many different fish species and areas, their morphology is very uniform, even at the ultrastructural level (McVicar et al. 1987), and hence it is difficult to believe that some X-cells are microsporidians and others not. The rRNA gene of the X-cell organism formed only a weak cluster with haplosporidian parasites, which are found solely in invertebrate hosts (Bradbury 1994). This suggests that the X-cell organism is a species distinct from any known group of the Protozoa. Analyses using other genes or the genetic data of related species are necessary to identify the precise taxonomic position of the X-cell organism.

In some papilloma-like lesions observed in the present study, X-cells were present only in the basal part of the epithelium, and constituted only a small part of the lesions. Most cells in these lesions were skin epithelial cells of the host fish, suggesting that the X-cell organism stimulates proliferation of the host epithelium, as suggested by Dawe (1981).

Because of the morphological uniformity of X-cells reported from all over the world in various fish species, it is very likely that all X-cells in fish pseudotumors are parasitic protozoans, as found in the present study. However, this needs to be confirmed by future studies. Even if all X-cells are parasitic protozoans, it seems plausible that there is more than 1 species of this organism, since the symptoms of X-cell disease can be divided into at least 3 types, depending on the species and on the tissues in which the lesions occur. Type 1 develops pseudotumors on the branchial lamellae, and is seen in the common dab (Diamant & McVicar 1987)

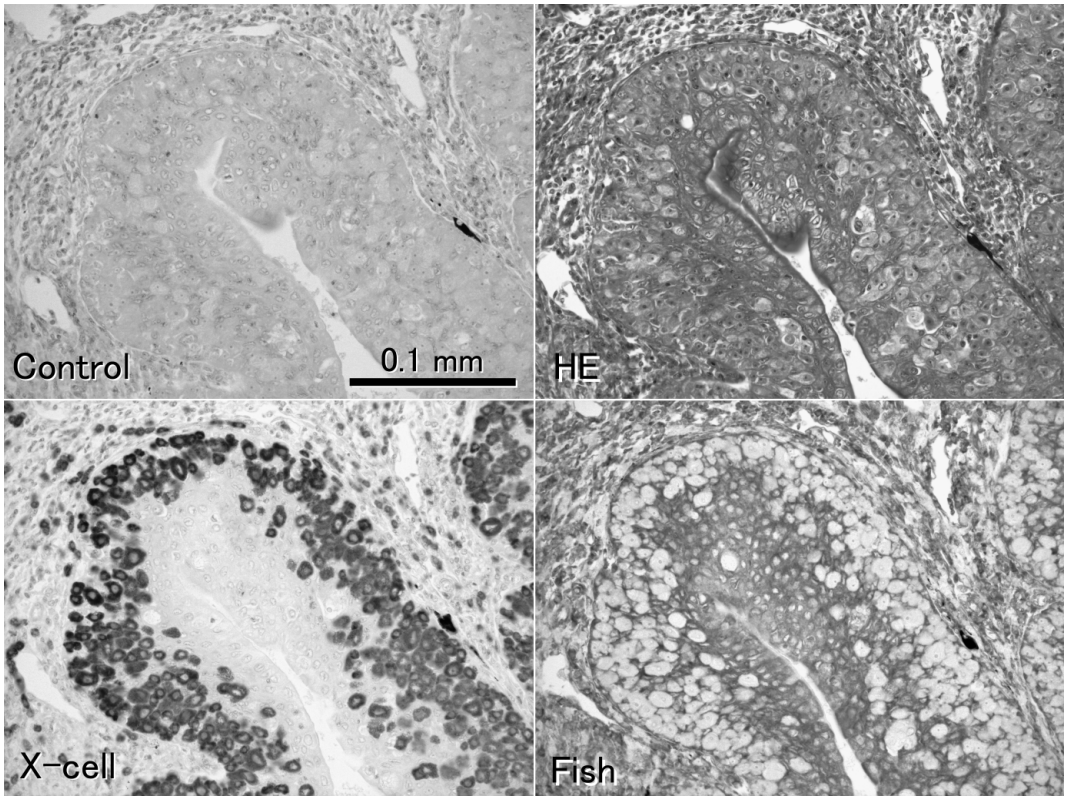

Fig. 3. *Hippoglossoides dubius*. Four succeeding sections of a papilloma-like X-cell lesion of a flathead flounder. Upper left: control section hybridized with combination of 4 oligoprobes specific for 16S rRNA of *Flavobacterium psychrophilum* bacterium; section was counterstained with hematoxylin. Upper right: hematoxylin-eosin (HE)-stained section. Lower left: location of protozoan 18S rRNA; section was hybridized with a combination of 3 oligoprobes (AXC1, AXC2, and AXC3) specific to the determined sequence; only X-cells, and no host-fish cells, contain this rRNA; small X-cells in the granulation tissue under the epithelium are also labelled. Lower right: section hybridized with a probe (TL1) specific to vertebrate 18S rRNA; probe hybridized only with host fish cells, and not with X-cells

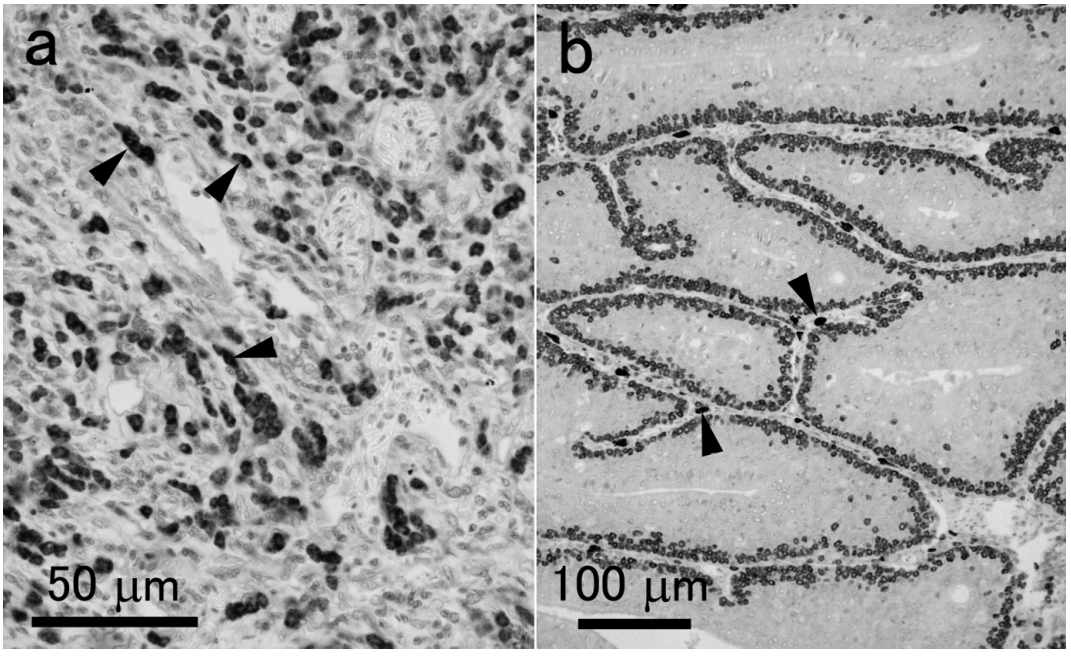

Fig. 4. *Hippoglossoides dubius*. Location of X-cells revealed by *in situ* hybridization with probes AXC1, AXC2, and AXC3. (a) Section of fibroma-like lesion, with many small X-cells (arrowheads) visible in granulation tissue. (b) Papilloma-like lesion, with X-cells visible only in basal part of proliferating skin epithelium, which forms complex papillae; arrowheads indicate melanophores

and a nototheniid fish in the Antarctic sea (Franklin et al. 1993); the common dab seems to be the only flatfish species in the Atlantic so far reported to be susceptible to X-cell disease (Diamant & McVicar 1990). Type 2 forms lesions primarily on the pseudobranch of cod and related species, both in the Atlantic (Dethlefsen et al. 1996) and in the Pacific (McCain et al. 1979). Type 3 develops lesions on the skin of fish body; this type has been reported for various species of flatfishes and a gobiid fish in the Pacific (Peters et al. 1978, Katsura et al. 1984) and in hardhead catfish in a lake in the USA (Diamant et al. 1994). Thus, X-cell organisms seem to have certain host-specificity or tissue-specificity. Furthermore, cell division or nuclear division of X-cells have been reported in the Pacific cod (Dawe 1981) and in the North Atlantic dab (Diamant & McVicar 1990), whereas such features have not been observed in X-cells of Pacific flatfish species (Peters et al. 1981, 1983).

Although X-cells are evidently parasitic protozoans, at least in the pseudotumors of flathead flounder, an attempt to transmit the disease by cell transplantation was unsuccessful (Wellings et al. 1976). This may be because the X-cell organism has a different life stage or a different host organism prior to fishes. If this is the case, another possible host or another possible life stage might be found using PCR or *in situ* hybridization and using the genetic data reported in this study.

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